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A preliminary study for evaluation of SARS-COV-2 RNA present on atherosclerotic plaques

SARS-CoV-2 on the atherosclerotic plaques

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Abstract

Aim: The aim of this study is to investigate the presence of SARS-CoV-2 RNA genome in atherosclerotic plaques in patients with a history of COVID-19 who are scheduled for surgery due to peripheral artery disease.

Material and Methods: Between the relevant dates, 40 patients with COVID-19 disease who were operated for peripheral artery disease in our clinic were included in the study. Of the 40 samples, 20 were aortic tissue and 20 were endothelial tissue. These tissues consist of samples taken from the patient at the Cardiovascular Surgery Department. The samples were transferred to the Microbiology laboratory and extracted by PCR procedure. Laboratory diagnosis with the kit was performed by multiplex RT-qPCR targeting SARS-CoV-2 ORF1ab and N genes in a real-time PCR device.

Results: Eight out of 20 patients in the endothelial group were female and 12 were male. Ten out of 20 patients in the aortic group were female and 10 were male. All samples were found as negative by PCR for SARS-CoV-2 at two study groups (aortic and endothelial tissues).

Discussion: This valuable study was conducted under limited resources and challenging pandemic conditions. We believe that this study will serve as a preliminary investigation shedding light on the effects of atherosclerotic plaques in patients with COVID-19 and PCR positivity in long-term and large-scale cases.

Keywords

Atherosclerotic Plaque, SARS-CoV-2, Real Time PCR, Peripheral Tissue, Vascular Tissue

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Introduction

Coronavirus disease 2019 (COVID-19) was declared a pandemic by the WHO on March 11, 2020 [WHO Director-General's opening remarks at the media briefing on COVID19 -March 2020]. In addition to respiratory failure, COVID-19 is associated with other life-threatening complications such as sepsis, heart failure, and pulmonary embolism [1]. Changes in blood coagulation during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, as described by Han et al., have been reported, including increases in D-dimer, fibrin or fibrinogen degradation products, and fibrinogen levels, as well as decreases in antithrombin levels, prothrombin time activity, and thrombin time. The systemic proinflammatory cytokine response mediates atherosclerosis by promoting the expression of procoagulant factors, local inflammation, and hemodynamic changes [2]. Atherosclerosis is a disease of chronic inflammation that eventually leads to tissue damage and fibrosis. The precise triggers of chronic inflammation are not clear, but lipid accumulation and alterations are thought to be significant components [3]. There is emerging evidence that infection is also a risk factor for atherosclerosis and contributes to chronic inflammatory processes through direct or indirect effects. Infectious agents can contribute to chronic inflammatory processes through direct or indirect mechanisms [4]. Direct effects can be determined by the ability of organisms to infect vascular cells, demonstration of the organism within atherosclerotic plaques, and accelerated lesion development in animal models of atherosclerosis following infection. Alternatively, the indirect effect of infectious agents resulting from an infection occurring in a non-vascular area is supported by increased cytokines and other acute-phase proteins, leading to accelerated atherosclerosis in experimental models. Numerous bacterial and viral pathogens have been detected in human atherosclerotic plaques or associated with cardiovascular disease through other means. Like other coronaviruses, SARS-CoV-2 also uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter target cells, but it has a higher affinity for ACE2 [5]. After binding to the ACE2 receptor, it activates the renin-angiotensin system (RAS), which leads to a decrease in ACE2 expression and consequently an increase in angiotensin II (Ang II) levels and a decrease in its counteracting peptide, angiotensin [6]. The RAS plays a significant role in COVID-19, and angiotensin-converting enzyme 2 (ACE2) acts as a functional receptor for SARS-CoV-2, leading to the regulation of ACE2 and higher expression of Ang II. ACE2 is primarily found in vascular endothelial cells of the lungs, but it is also present in extrapulmonary tissues, such as the heart, nervous system, intestines, kidneys, blood vessels, and muscle cell surfaces. Recent studies examining organ involvement associated with COVID-19 have shown that COVID-19 comorbidities increase the frequency of thrombotic events, particularly deep vein thrombosis and pulmonary embolism [7]. However, while coronary, cerebrovascular, and peripheral arterial thrombotic events have been demonstrated in small series, their true incidence, outcomes, and effectiveness of SARS-CoV-2 in COVID-19 have not been well defined [8]. The aim of this study is to investigate the presence of the SARS-CoV-2 RNA genome in atherosclerotic plaques in patients with a history of COVID-19 who are scheduled for surgery due to peripheral artery disease.

Material and Methods

The research was conducted in accordance with the World Medical Association Declaration of Helsinki. This study protocol was reviewed and approved by the Ethics Committee of Namık Kemal University, Medical Faculty, Tekirdag / Turkey (Approval No: 2022.171.09.18, Approval date: 2022-09-18).

Subjects

The study included patients with COVID-19 who underwent surgery for peripheral artery disease in our clinic from 01.09.2021 to 01.05.2022. Demographic characteristics, comorbidities and anatomical data of the patients were evaluated as preoperative data. Specifically, age, gender, coronary artery disease (CAD), renal failure, smoking history, previous stroke, or transient ischemic attack (TIA), pulmonary comorbidities, and hypertension were examined. Tissue samples were taken during the operation and stored under cold chain conditions at -20°C. Then, the genomic structure (nucleic acid) of the SARS-CoV-2 virus was investigated by PCR technique in the samples of these tissues in the microbiology laboratory. It was investigated whether the SARS-CoV-2 virus was present in peripheral vascular tissues of people who were diagnosed with COVID-19 disease or had the disease by molecular study. In addition, detailed operative and postoperative data were obtained from the files of the patients and included in the study. In the study, which was carried out on a voluntary basis, complications that developed in the operative and postoperative period in the patients were questioned and recorded. The study was designed to be both retrospective and prospective. Statistical comparative analyzes of the data obtained after the study were carried out.

Sample Collection, Transportation, and Storage

Between the relevant dates, 40 patients with COVID-19 disease who were operated for peripheral artery disease in our clinic were included in the study. Patients who did not have peripheral artery disease or whose operation was contraindicated were not included in the study. Of the 40 samples, 20 were aortic tissue and 20 were endothelial tissue. These tissues consisted of samples taken from the patient at Tekirdag Namık Kemal University, Faculty of Medicine Cardiovascular Surgery Department with the consent of the patient and rendered inactive. After these samples were taken from the patient, they were kept in physiological saline at -20°C until the day of the experiment. After the samples were collected, they were transferred to the laboratory of the Microbiology Department with the cold chain and appropriate transportation conditions and kept until the day of the experiment.

Sample Preparation for q-RT-PCR Tests

The Bio-Speedy Rapid Nucleic Acid Extraction Kit was used for nucleic acid extraction and purification. This system is a magnetic bead purification based robotic nucleic acid extraction system. This kit is used for in-vitro diagnostics based on clinical samples. Transport and storage conditions for these samples complied with national and international transport standards. The samples were thawed after being taken at -20°C. Pea-sized tissue samples were cut into small pieces in a sterile container with the help of a scalpel. The fragmented sample and 20 µL of "5 min NA" included in the rapid extraction kit were transferred to the "STL-B tube" and vortexed for 10 seconds at maximum

speed. Then, it was incubated at 95 °C for 3 minutes and then vortexed again for 10 seconds at maximum speed. Zybio EXM3000 device (Zybio, China) was used for robotic extraction process. After the device was ready for use, the samples were placed on the device stand in the specified order. 15 µL of Proteinase K was pipetted into the well-1 of the cartridge. 200 µL of clinical sample was pipetted into the same well, and than device was started. At the end of the procedure, 40-50 µL of isolate in well 6 of the cartridges was transferred to 1.5 mL Eppendorf nuclease-free with a micropipette. This sample was ready for q-RT-PCR study.

Bio-Speedy SARS-CoV-2 Double Gene RT-qPCR Kit and RT-qPCR Test interpretation

It is a one-step reverse transcription and real-time PCR test designed for the qualitative detection of SARS-CoV-2 RNA. Laboratory diagnosis with the kit was performed by multiplex RT-qPCR targeting SARS-CoV-2 ORF1ab and N genes according to the kit protocol in a real-time PCR device (Bio-RadLaboratories, Inc. USA) with FAM and HEX channels. If Cq< 33 in the FAM channel, the result is recorded as positive. Non-sigmoidal curves are considered negative.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

When the demographic data of the patients were examined, 8 out of 20 patients in the endothelial group were female and 12 were male. The average age of women was 63 years, and their age range was between 57 and 80. BMI ranged from 17.6 to 29.4 with an average of 22.9. In men, the average age was 60.8 years, and the age ranged from 44 to 75 years. The BMI ranged from 17.5 to 31.1 with an average of 23.9. Thus, when considering the demographic characteristics of the total endothelial group, the average age was 61.7 years, ranging from 44 to 80 years. The BMI range of the total endothelial group was between 17.5 and 31.1, with an average of 23.5. Ten out of 20 patients in the aortic group were female and 10 were male. The average age of women was 65,4 years and their age ranged from 43 to 78. BMI ranged from 19.0 to 29.0 with an average of 24,6. In men, the average age was 65.0 years, and the age ranged from 32 to 91 years. The BMI range was between 18.1 and 29.2 with an average of 25.2. Thus, when considering the demographic characteristics of the total aortic group, the average age was 65.6 years, ranging from 32 to 91 years. The BMI range of the total aortic group was between 18.6 and 29.2, with an average of 24.9 (Tables 1, 2, 3).

RT-PCR Results

Samples with Cq_FAM values below 35 tend to be positive. The numerical value of these two samples was positive, but when the sigmoidal curve of these two samples was examined according to the kit protocol, there was no sigmoidal curve like the positive control samples. Therefore, these samples were interpreted as negative according to the kit protocol (Figure 1). Looking at Figure 1, it can be seen that the negative control and internal control used for the detection of contaminated material are working correctly. The internal control HEX channels also worked normally according to the kit protocol. Thus, the study was performed correctly according to the kit protocol. All samples were found negative by PCR for SARS-CoV-2 in two study groups (aortic and endothelial tissues).

Table 1. Age, gender, and BMI characteristics in the aortic and endothelial tissue group.

Specimen Endothelial (n:20) Aortic (n:20)	Parameters	Range	Average	P
Endothelial Woman (8/20)	AGE (years)	57-80	63	p>0.05
	BMI (İndex)	17.6-29.4	22,9	
Endothelial Man (12/20)	AGE	44-75	60,8	
	BMI	17.5-31.1	23,9	
Aortic Woman (10/20)	AGE	43-78	65,4	p>0.05
	BMI	19.0-29.0	24,6	
Aortic Man (10/20)	AGE	32-91	65	
	BMI	18.1-29.2	25,2	
Endothelial Total (20/20)	AGE	44-80	61,7	p>0.05
	BMI	17.5-31.1	23,5	
Aortic Total (20/20)	AGE	32-91	65,6	p>0.05
	BMI	18.6-29.2	24,9	

BMI: Body mass index p: Chi-square test.

Table 2. Distribution of risk factors for atherosclerosis.

Specimen	Chronic Renal Failure		Hypertension		Hyperlipidemia		Chronic Obstructive Pulmonary Disease		Diabetes Mellitus		Smoke		Alcohol		Heart Failure	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Endothelial Woman (8/20)	1	12,5	2	25	3	37,5	0	0	3	37,5	0	0	0	0	0	0
Endothelial Man (12/20)	1	8,5	4	33,3	8	66,6	3	24,9	4	33,3	7	58,3	5	41,6	2	16,6
Endothelial Total (20/20)	2	10	6	30	11	55	3	15	7	35	7	35	5	25	2	10
Aortic Woman (10/20)	3	30	8	80	2	20	2	20	2	20	5	50	0	0	0	0
Aortic Man (10/20)	2	20	5	50	2	20	3	30	2	20	7	70	6	60	2	20
Aortic Total (20/20)	5	25	13	65	4	20	5	25	4	20	12	60	6	30	2	10
Total (40/40)	7	17,5	19	47,5	15	37,5	8	20	11	27,5	19	47,5	12	30	4	10

Table 3. Distribution of PCR results in groups according to the vaccine protocol applied.

Specimen	Inactive-1		Inactive-2		mRNA-1		mRNA-2		PCR History		COVID-19 History	
	n	%	n	%	n	%	n	%	n	%	n	%
Endothelial Woman (8/20)	4	50	3	37,5	2	25	4	50	0	0	0	0
Endothelial Man (12/20)	2	16,6	10	83,3	11	91,6	0	0	1	8,3	0	0
Endothelial Total (20/20)	6	30	13	65	13	65	4	20	1	5	0	0
Aortic Woman (10/20)	3	30	6	60	5	50	3	30	1	10	0	0
Aortic Man (10/20)	3	30	7	70	8	80	0	0	1	10	0	0
Aortic Total (20/20)	6	30	13	65	13	65	3	15	2	10	0	0
Total (40/40)	12	30	26	65	26	65	7	17,5	3	7,5	0	0

Discussion

Atherosclerosis is a disease that occurs in the inner lining of the blood vessels due to the influence of various factors. Although ideas about the infectious basis of atherosclerosis were proposed over a century ago, the first experimental evidence that infection could induce atherosclerotic changes was reported by Fabricant et al., who demonstrated that Marek's disease virus (MDV), a chicken herpesvirus, could induce atherosclerosis in chickens [9]. Since these initial studies, respiratory pathogens (such as C. pneumoniae and influenza viruses), periodontal pathogens (P. gingivalis, A. actinomycetemcomitans), a gastric pathogen (H. pylori), and cytomegalovirus, a common cause of congenital and perinatal infections, have been reported to accelerate the progression of atherosclerotic lesions. They have provided evidence of a biological role in atherosclerosis pathology in animal models. Various pathogens have been identified in human atherosclerotic plaques using nucleic acid or antigen detection methods [10]. Examples of bacterial pathogens include Chlamydia pneumoniae (C. pneumoniae), Mycoplasma pneumoniae, Helicobacter pylori, Enterobacter hormaechei, and multiple periodontal organisms (such as Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia, Fusobacterium nucleatum, Streptococcus sanguis, and Streptococcus mutans) [11]. Examples of viral pathogens include cytomegalovirus, hepatitis C virus, human immunodeficiency virus, herpes simplex viruses, Epstein-Barr virus, enteroviruses, and parvovirus [12]. In some studies, the presence of multiple infectious agents within atherosclerotic tissue has been reported [13]. It is known that SARS-CoV-2, an RNA virus, can enter the cell cytoplasm and cause tissue damage, potentially leading to chronic infection. Recently, it has been emphasized that thrombotic events contribute to the severity of the disease in patients with SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) infection [14], and antithrombotic treatment is progressively expanding worldwide [15], An increased incidence of pulmonary embolism has been reported [16], and cardiac involvement with elevated markers of myocardial damage has been found in 10-20% of cases [17]. Although small patient series have reported coronary, cerebrovascular and peripheral arterial events, the true incidence and clinical outcomes of these complications have not been well established. Influenza infection, a single-stranded RNA virus, is associated with acute coronary syndrome and fatal myocardial infarction [18]. Although studies in mice have detected the virus in the vascular wall, it has not been investigated whether influenza infection accelerates the progression of atherosclerotic lesions in this animal model. However, influenza infection is generally believed to be limited to the pulmonary system in humans, and there are no reports of the organism being detected in human atherosclerotic plaques. Therefore, there is no supporting evidence for a direct effect of influenza viruses on the pathogenesis of atherosclerosis in humans [19]. Several studies have shown that acute respiratory tract infections can serve as triggers for myocardial infarction [20]. Therefore, the hypothesis that influenza infection may play a role in complications of atherosclerosis was tested, and elderly apoEe/e mice with atherosclerosis were infected, and histopathological changes in the atherosclerotic lesion in the aorta were assessed at 3, 5, and 10 days post-infection [21]. The LD50 dose of the virus was administered intranasally to the mice. Infected apoEe/e mice exhibited significantly increased subendothelial cellular infiltrations compared to uninfected apoEe/e mice and infected C57BL/6 mice with normal lipid levels. These infiltrations consisted of smooth muscle cells, macrophages, and T lymphocytes. Importantly, a subocclusive thrombus rich in platelets and fibrin was observed in one infected mouse. Notably, no histopathological changes were observed in the non-atherosclerotic region of the infected aorta. Influenza A infection in mice also leads to the loss of the anti-inflammatory properties of HDL, which may be another mechanism by which the virus can indirectly impact the progression of atherosclerotic lesions [22]. Finally, angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, is expressed in myocytes and vascular endothelial cells, suggesting at least a theoretical possibility of direct cardiac involvement by the virus [23].

Conclusion

Given the young and seemingly healthy patients who develop severe vascular complications during SARS-CoV-2 infection, it is essential to create a prospective registry to understand the prevalence and risk factors of acute limb ischemia in COVID-19 patients, in order to identify prophylactic and therapeutic protocols. Research on the role of infection in atherosclerosis is at a turning point. Despite the presence of various infectious agents in human atherosclerotic plaques and the causality of atherosclerotic plaque progression demonstrated in animal models, many unanswered questions remain regarding whether infectious agents contribute to cardiovascular disease, which

is considered a risk factor. This study aimed to investigate the presence of the SARS-CoV-2 RNA genome in atherosclerotic plaques during the pandemic. For this purpose, we aimed to examine the genomic structure of the virus using PCR in atherosclerotic plaque samples obtained intraoperatively from patients scheduled for surgery due to peripheral artery disease, in order to determine if they had COVID-19. As known, 85% of SARS-CoV-2 infections are asymptomatic, and 10-15% of them present to clinics [24]. There were no positive PCR results among the 40 patients included in this study. Although limited resources and challenging conditions during the pandemic prevented a satisfactory number of patients included in this study, we believe that this study will serve as a preliminary investigation shedding light on the effects of atherosclerotic plaques in patients with COVID-19 and PCR positivity in long-term and large-scale cases.

Scientific Responsibility Statement
The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of Interest
The authors declare no conflict of interest.

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